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(71) Applicant *(for all designated States except US)*: **HWANG, Woo-Suk** [KR/KR]; 11-2 Nonhyun-dong, Kangnam-gu, Seoul 135-010 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LEE, Byeong-Chun** [KR/KR]; 106 Hoamdongkwan, 239-1 Bongcheon-dong, Kwanak-gu, Seoul 151-050 (KR). **SHIN, Tae-Young** [KR/KR]; 148-91 Bongcheon 6-dong, Kwanak-gu, Seoul 151-056 (KR). **ROH, Sang-Ho** [KR/KR]; 205-601 Daewoo Kyungnam Apartment, Dangwang-dong, Ansung-shi, Kyunggi-do 456-360 (KR). **LIM, Jeong-Muk** [KR/KR]; 1-601 Miseong Apartment, 219 Poongnap-dong, Songpa-gu, Seoul 138-040 (KR). **PARK, Jong-Im** [KR/KR]; 29-1 Sangdo 2-dong, Dongjak-gu, Seoul 156-032 (KR). **CHO, Jong-Ki** [KR/KR]; 230-4 Seongnae 2-dong, Kangdong-gu, Seoul 134-032 (KR). **KIM, Ki-Yon** [KR/KR]; 1205 Konyoungshilim 5 cha Apartment, Shilim 9-dong, Kwanak-gu, Seoul 151-019 (KR). **LEE, Eun-Song** [KR/KR]; 205-601 Soksa Daewoo Apartment, 721-2 Soksa-dong, Chooncheon-shi, Kangwon-do 200-180 (KR). **SHIN, Soo-Jung** [KR/KR]; 643-13 Jayang 2-dong, Kwangjin-gu, Seoul 143-192 (KR). **KIM, Sung-Ki** [KR/KR]; 1-302 Hyangnam Apartment, Bangchook-ri, Hyangnam-myun, Hwasung-gun, Kyunggi-do 445-920 (KR). **HAN, Jae-Yong** [KR/KR]; 101-513 Yongin 3 cha Dongbo Apartment, 504-10 Poongdukchon-ri, Sooji-eup, Yongin-shi, Kyunggi-do 449-840 (KR). **YONG, Hwan-Yul** [KR/KR]; 85-1 Songjook-dong, Changan-gu, Suwon-shi, Kyunggi-do 440-210 (KR). **CHOI, Yun-Hee** [KR/KR]; 102-1306 Hyosong Town, Bongdeok 2-dong, Nam-gu, Taegu 705-022 (KR). **KO, Bong-Kyung** [KR/KR]; 603-87 Chang 2-dong, Dobong-gu, Seoul 132-042 (KR). **SONG, Kil-Young**

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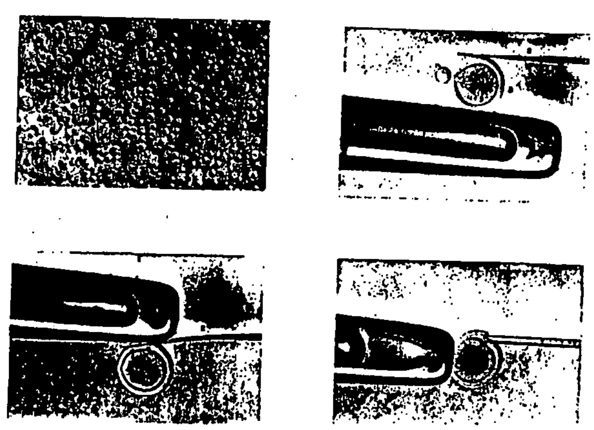
(54) Title: METHOD FOR PRODUCING HUMAN CLONED EMBRYOS BY EMPLOYING INTER-SPECIES NUCLEAR TRANSPLANTATION TECHNIQUE



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(57) Abstract: The present invention provides a method for producing human cloned embryos by employing inter-species nuclear transplantation technique. The method for producing human cloned embryos of the invention comprises the steps of: preparing donor somatic cell lines collected from human; maturing oocytes collected from ovary of cow in vitro; removing the cumulus cells surrounding the oocytes; cutting a portion of zona pellucida of the matured oocytes to make a slit, and squeezing out a portion of cytoplasm including the first polar body through the slit to give enucleated recipient oocytes; transferring a nucleus to the recipient oocyte by injection of the donor cells to the enucleated recipient oocytes, followed by the subsequent electrofusion and activation of the electrofused cells to give embryos; and, postactivating and culturing the embryos in vitro. The human cloned embryos of the invention can be employed to obtain the human embryonic stem cells, which may be widely applied in biological and medical fields.

[KR/KR]; 70-41 Myonmok 6-dong, Joongrang-gu, Seoul 131-206 (KR).

(74) **Agent:** **LEE, Han-Young**; 8th Fl., Seowon Building, 1675-1 Seocho-dong, Seocho-gu, Seoul 137-070 (KR).

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Method For Producing Human Cloned Embryos By Employing Inter-species Nuclear Transplantation Technique

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method for producing human cloned embryos by employing inter-species nuclear transplantation technique, more specifically, a method for producing human cloned embryos by employing inter-species nuclear transplantation technique by which nuclei of somatic cells derived from human tissue are transferred into mature oocytes originated from cow. It also relates to human cloned embryos produced by the method described above.

Backgound of the Invention

Animals have long been considered to be produced by fertilization involving male and female gametes. However, tremendous efforts have been made on generating cloned animals with the identical appearance and genetic characteristics.

Recently, various recombinant plants with desired characters of useful crops have been successfully produced due to the developments in biotechnology and genetic engineering(see: Schweizer et al., Plant Journal, 20:541-552, 1999). From animal's side, there have been many successful examples in production of cloned animals which include, for example, cloned lambs(see: Wilmut et al., Nature, 385:810-813, 1997), cloned cows(see: Wells et al., Reprod. Fertil. and Develop., 10:369-378, 1998) and cloned mice(see: Wakayama et al., Nature, 394:369-374, 1998). Since production of cloned animals cannot be realized without high technology built up on the biotechnology, it

has been considered to be a standard to assess the technological development in the related fields.

Meanwhile, animal stem cells have been known to have potency of development to every organ, which prompted the research on the mechanism of their differentiation to each organ by obtaining and culturing them. When this research is conducted, it is important to employ materials with the identical tissue-specificity to reduce variations among many different studies. However, it is obvious that suitable materials with the identical tissue-specificity are not available all the time. Although the cloning technique employing somatic cells has facilitated the supply of materials with the identical tissue-specificity recently, it was less satisfactory in case of human tissue.

Under the circumstances, there have been strong reasons for developing a method for obtaining human embryonic stem cells with the identical tissue-specificity.

Summary of the Invention

In accordance with the present invention, it has been discovered that: human embryonic stem cells can be successfully produced by inter-species nuclear transplantation technique involving fusion of oocytes of cow and human skin cells and culturing the human cloned embryos in vitro to the stage of morulae/blastocysts.

A primary object of the present invention is, therefore, to provide a method for producing human cloned embryos by inter-species nuclear transplantation technique.

The other object of the invention is to provide human cloned embryos produced by the said method.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and the other objects and features of the present invention will become apparent from the following

description given in the conjunction with the accompanying drawings, in which:

Figure 1 is a photograph of donor somatic cells.

Figure 2 is a photograph showing the process of cutting zona pellucida of a recipient oocyte with a holding pipette and cutting pipette.

Figure 3 is a photograph showing the process of enucleation by removing the first polar body and nucleus from a recipient oocyte.

Figure 4 is a photograph showing the process of transferring a somatic cell into an enucleated oocyte with a holding pipette and injection pipette.

DETAILED DESCRIPTION OF THE INVENTION

The method for producing human cloned embryos of present invention comprises the steps of: preparing donor somatic cell lines collected from human; maturing oocytes collected from ovary of cow in vitro; removing cumulus cells surrounding the oocytes, cutting a portion of zona pellucida of the matured oocytes and squeezing out a portion of cytoplasm including the first polar body to give enucleated recipient oocytes; transferring a nucleus to the recipient oocyte by injection of the donor cells to the enucleated oocytes, followed by the subsequent electrofusion and activation of the electrofused cells to give embryos; postactivating and culturing the embryos in vitro.

The method for producing human cloned embryos of the invention is further illustrated as follows.

Step 1: Preparation of donor cells

Somatic cell lines collected from human are prepared as donor cells: although cells collected from human are not limited for donor cells, preferable cell lines include skin cells or fibroblasts collected from the umbilical cord of newborns. The more preferable cell line for donor cell is skin cells isolated from skin tissue. The said cell lines are prepared by employing the conventionally known method(see: Mather & Barnes, Methods in Cell Biology, Vol.57, Animal Cell Culture Methods, Academic Press, 1998) with some modifications.

For example, skin cells or fibroblasts of newborn's umbilical cord are washed and minced. Then, the cells are subjected to treatment of trypsin and collagenase type II under an environment of 39℃, 5% CO_2, followed by culture in DMEM(Dulbecco's modified Eagle's medium) supplemented with non-essential amino acids, 10% FBS(fetal bovine serum) and 1% penicillin-streptomycin(10000U/ml penicillin, 10 mg/ml streptomycin) under the same environment described above.

The somatic cell lines are stored by subculture, serum starvation culture or freezing. The subculture of donor cell lines is carried out at regular intervals by changing the old medium to new one after trypsinization. The serum starvation culture is performed by employing DMEM supplemented with 0.5% FBS and the method of Wilmut et al.(see: Wilmut et al., Nature, 385:810-813, 1997). The cell lines thus stored are used for later step as donor cells.

Step 2: Preparation of recipient oocytes

Immature oocytes collected from ovary of cow are matured in vitro: immature oocytes are selected from ovary in TCM199 washing medium containing 10 mM HEPES(N-[hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), and matured by culturing the cells in TCM199 culture

medium(containing Na-pyruvate, penicillin-streptomycin) supplemented with estradiol, FSH(follicle stimulating hormone) and FBS for 16 to 22hr under an environment of 39℃, 5% CO_2.

<u>Step 3</u>: Enucleation of recipient oocytes

After removing cumulus cells surrounding the mature recipient oocytes and cutting a portion of zona pellucida of the oocytes, a portion of cytoplasm including the first polar body is removed from the oocytes to give enucleated oocytes: first, cumulus cells surrounding the mature oocytes are removed physically with a denuding pipette in TCM199 washing medium containing hyaluronidase. Then, denuded oocytes are washed with TCM199 washing medium and transferred into cytochalasin B solution. For enucleation of the denuded oocytes, a portion of zona pellucida of the denuded oocytes is penetrated by a cutting pipette to give a slit through which 10 to 15 % of cytoplasm including the first polar body can be squeezed out of the oocytes. The enucleated oocytes are washed and incubated in TCM199 culture medium. The said cytochalasin B solution is prepared by diluting cytochalasin B dissolved in DMSO(dimethylsulfoxide) with the TCM199 culture medium.

<u>Step 4</u>: Electrofusion of donor cells with recipient oocytes and activation of the electrofused cells

The donor cells are transferred to the recipient oocytes, followed by subsequent electrofusion and activation of the electrofused cells: before the injection of donor cells into recipient oocytes, the enucleated oocytes are washed with TCM199 culture medium and transferred to PHA-P(phytohemagglutinin) solution. Then, the donor cells are transferred to the enucleated oocytes by injecting donor cells to the slit made on zona pellucida of the oocytes in PHA-P solution.

The electrofusion is carried out by employing Electro Cell Manipulator(BTX ECM2001). The reconstructed embryos in mannitol solution supplemented with TCM199 washing solution are placed in a chamber with two electrodes, one on either side. Before placing the embryos with their donor cells facing the cathode in the chamber, the chamber was filled with mannitol solution. After the embryos are electrofused by applying DC pulse of 0.75 to 2.00 kV/cm twice with one second's interval for 15μs each time, the electrofused embryos are washed with mannitol solution and TCM199 washing medium, incubated in cytochalasin B solution, and activated. The electrofusion and activation occur in a simultaneous manner provided that the electrofusion is carried out in a mannitol medium containing Ca^{2+}. Otherwise, the activation is performed after electrofusion. When the electrofusion is carried out in a Ca^{2+}-free mannitol medium, the activation step is performed by incubating the embryos in ionomycin solution in the dark. Then, ionomycin is removed from the embryos by washing them with TCM199 washing medium containing FBS or BSA. The said ionomycin solution is prepared by diluting ionomycin dissolved in DMSO with TCM199 washing medium containing BSA.

<u>Step 5</u>: Postactivation and in vitro culture of embryos

The embryos are postactivated and cultured in vitro: the activated embryos incubated in TCM199 washing medium containing FBS or BSA are postactivated by incubating in cycloheximide solution or DAMP(4-dimethylaminopurine) solution, and cultured in vitro under an environment of 5% CO_2, or a mixture of 5% CO_2, 7% O_2 and 88% N_2. The said cycloheximide solution or DAMP solution is prepared by adding cycloheximide dissolved in ethanol or DAMP to media for in vitro culture, respectively. The media for in vitro culture include mTALP(<u>see</u>: Table 1), mSOF(<u>see</u>: Table 2) and mCR2aa(<u>see</u>: Table 3) medium, all of which comprise NaCl, KCl, $NaHCO_3$, NaH_2PO_4, $CaCl_2$, Na-lactate, glucose, phenol red,

BSA, kanamycin, essential amino acids, non-essential amino acids and L-glutamine.

Optionally, the embryos cultured in vitro are stored by freezing for later use, and subjected to thawing when they are intended to be used. To freeze the embryos, they are washed with PBS containing FBS, put in a freezing medium containing penicillin-streptomycin, $CaCl_2$, glucose, $MgCl_2$, Na-pyruvate and PBS. Then, the embryos in the freezing medium are subjected to slow freezing, followed by rapid freezing in liquid N_2. When the frozen embryos are taken from liquid N_2 and thawed, they are put in the air for about 5 seconds and then thawed in warm water. To remove the freezing medium from the thawed embryos, they are put serially in media containing glycerol from its high concentration to low concentration.

Table 1: mTALP medium

Ingredient	Concentration
NaCl	93.1~103.4mM
KCl	3.1mM
$NaHCO_3$	25mM
NaH_2PO_4	0.36mM
Na-lactate	15mM
$CaCl_2 \cdot 2H_2O$	1.7mM
$MgCl_2 \cdot 6H_2O$	0.5mM
Na-pyruvate	0.45mM
Glucose	1.5mM
Phenol red	10 μg/ℓ
BSA	8 mg/mℓ
Kanamycin	0.75 μg/mℓ
EAA(essential amino acids)	2%
NEAA(non-essential amino acids)	1%
L-glutamine	1mM
ITS(insulin-transferrin-sodium selenite media supplement)	0.5%

Table 2: mSOF medium

Ingredient	Concentration
NaCl	99.1~106mM
KCl	7.2mM
$NaHCO_3$	25mM
NaH_2PO_4	1.2mM
Na-lactate	5mM
$CaCl_2 \cdot 2H_2O$	1.7mM
$MgCl_2 \cdot 6H_2O$	0.5mM
Na-pyruvate	0.3mM
Glucose	1.5mM
Phenol red	10 μg/ℓ
BSA	8 mg/mℓ
Kanamycin	0.75 μg/mℓ
EAA(essential amino acids)	2%
NEAA(non-essential amino acids)	1%
L-glutamine	1mM
ITS	0.5%

Table 3: mCR2aa medium

Ingredient	1st Culture medium (1-4days)	2nd Culture medium (After 4th day)	Washing medium
NaCl	114mM	114mM	114mM
KCl	3.1mM	3.1mM	3.1mM
$NaHCO_3$	25mM	25mM	2mM
NaH_2PO_4	0.35mM	0.35mM	0.34mM
Na-lactate	15mM	15mM	15mM
$CaCl_2 \cdot 2H_2O$	2mM	-	2mM
$MgCl_2 \cdot 6H_2O$	0.5mM	0.5mM	0.5mM
EAA	-	1%	-
NEAA	1%	1%	1%
Insulin	1%	1%	1%
Glutamine	-	1mM	1mM
Glycine	0.37mM	0.37mM	0.37mM
Citric acid	0.33mM	0.33mM	0.33mM
HEPES	-	-	10.5mM
Na-pyruvate	0.3mM	0.3mM	-
Glucose	-	1.5mM	-
Phenol red	10 μg/ℓ	10 μg/ℓ	10 μg/ℓ
BSA	3 mg/mℓ	-	3/mℓ
FBS	-	10%	-
Kanamycin	0.75 μg/mℓ	0.75 μg/mℓ	0.75 μg/mℓ

Based on the method described above, the present inventors produced an embryo, SNU6(human somatic cell line),

by using human skin cells as nucleus donors. The embryo was deposited with an international depositary authority, KCTC(Korean Collection for Type Cultures; KRIBB #52, Oun-dong, Yusong-ku, Taejon, 305-333, Republic of Korea) on June 19, 2000 under an accession number of KCTC 0805BP.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Preparation of donor cells and recipient oocytes

To prepare donor cells, tissue collected from human skin was washed with PBS(phosphate buffered saline, Gibco BRL, Life Technologies, USA) and minced into 100 mesh size. Then, the tissue was incubated in PBS containing 0.25% trypsin, 1mM EDTA and 1mg/ml collagenase type II for 1hr under an environment of 39℃, 5% CO_2. After the tissue was digested with the enzymes, it was centrifuged at 1,500 rpm for 2 minutes, and suspended in DMEM(Dulbecco's modified Eagle's medium, Gibco BRL, Life Technologies, USA) supplemented with 10% FBS, 1% NEAA(non-essential amino acids) and 1% penicillin-streptomycin. The suspension was transferred to dishes for cell culture and incubated under an environment of 39℃, 5% CO_2 to give a somatic cell line. After that, the cells were trypsinized in solution containing 0.25% trypsin and 1mM EDTA, and the cell number was adjusted to be 2 X 10^4 cells/ml to aliquot the cells in effendorf-tubes.

Figure 1 depicts the somatic cells isolated as single cells for nucleus donor.

On the other hand, for recipient oocytes, follicles of which size was 2 to 6 mm in diameter were aspirated from ovaries of Korean cows with a 10ml syringe having an 18G needle. Then, the follicular fluid were transferred into a 100mm dish with a grid(the length between lines was 1 cm) drawn on its bottom, and oocytes with homogeneous cytoplasm

and sufficient number of cumulus cell layers around them were screened. The selected oocytes were washed three times with 2 ml of TCM199 washing medium(see: Table 4) in 35mm dishes, and subsequently, once with TCM199 culture medium(see: Table 5). Finally, the oocytes were cultured in TCM199 culture medium containing 0.1% estradiol solution(see: Table 6), 2.5% follicle stimulating hormone solution(see: Table 7) and 10% FBS to give recipient oocytes.

Table 4: TCM199 washing medium

Ingredient	Concentration
TCM powder	Gibco 31100-027
HEPES	10mM
$NaHCO_3$	2mM
BSA	0.5% W/V
Penicillin-streptomycin	1% (penicillin 10000U/ml, streptomycin 10mg/ml)

Table 5: TCM199 culture medium

Ingredient	Concentration
TCM liquid	Gibco 11150-059
Na-pyruvate	1mM
Penicillin-streptomycin	1% (penicillin 10000U/ml, streptomycin 10mg/ml)

Table 6: Estradiol solution

Ingredient	Concentration
Estradiol	5mg
Ethanol	10ml

Table 7: Follicle stimulating hormone solution

Ingredient	Concentration
Follicle stimulating hormone	2AU
TCM199 culture medium	10ml

Example 2: Nuclear transfer of somatic cells

The recipient oocytes prepared in Example 1 were washed once with TCM199 washing medium and transferred in 0.1% hyaluronidase(Sigma Chemical Co., U.S.A.) solution prepared by mixing 1ml of TCM199 washing medium with 111μl of hyaluronidase stock solution(10mg/ml in TCM199 washing medium). After cumulus cells were removed from the oocytes in the presence of 0.1% hyaluronidase, the denuded oocytes were washed three times and incubated in TCM199 washing medium. Then, the oocytes were transferred to cytochalasin B(Sigma Chemical Co., U.S.A.) solution prepared by mixing 1ml of TCM199 washing medium containing 10% FBS with 1μl of cytochalasin stock solution(7.5mg/ml in DMSO), and zona pellucida of each oocyte was cut by employing micromanipulator to make a slit through which 10 to 15% of cytoplasm can be squeezed out of the oocyte to give an enucleated oocyte. The enucleation step is more specifically illustrated as following: a working dish was put on the micromanipulator plate, and the micromanipulator was equipped with a holding pipette on its left arm and a cutting pipette on its right arm. Then, the holding pipette and cutting pipette were placed in the direction of 9 o'clock and 3 o'clock, respectively, and adjusted to move freely in all directions by placing a pipette controller in the middle. These two pipettes were further adjusted to let them not touch the working dish and their tips placed to the middle of a microdroplet by moving them up and down over the microdroplet. Then, the oocytes were transferred from TCM199 washing medium to cytochalasin B solution by employing washing mouth pipettes(>200μm inner diameter). The micromanipulator was first focused on the oocyte by using its coarse adjustment knob and fine adjustment knob, and the focus was further adjusted by moving the two pipettes up and down. The oocyte was placed with its first polar body oriented toward the direction of 12 o'clock, and the holding pipette was placed close to the oocyte in the direction of 9 o'clock of the oocyte to fix the oocyte by applying hydraulic pressure. Figure 2 shows the process of

cutting zona pellucida of the oocyte with the holding pipette and cutting pipette. As shown in Figure 2, the oocyte was penetrated by the cutting pipette(2) from the direction of 1 o'clock to the direction of 11 o'clock with special care not to damage the cytoplasm of the oocyte. After that, hydraulic pressure was applied to the holding pipette(1) to separate the oocyte(3), and the holding pipette was contacted with the cutting pipette penetrating the zona pellucida bordering on the upper part of the first polar body to cut the portion of zona pellucida by rubbing the two pipettes. The slit on the oocyte made above was used for both enucleation and donor cell injection. Figure 3 depicts the process of enucleation removing the first polar body and nucleus from the oocyte. As shown in Figure 3, the oocyte(3) was placed with its slit oriented vertically, held with the holding pipette(1) on its lower part to prevent it from moving, and squeezed mildly on its upper part with the cutting pipette(2) to give an enucleated oocyte. The enucleated oocyte was washed three times with TCM199 washing medium and incubated in TCM199 culture medium.

After that, donor cells prepared in advance were transferred to enucleated oocytes by employing micromanipulator. First, 4μl of injection microdroplet was made on the middle of the working dish by using PHA-P solution prepared by mixing 400μl of TCM199 washing solution and 100μl of PHA-P(phytohemagglutinin) stock solution(0.5mg/ml in TCM199 washing solution). And then, two microdroplets for donor cells were made with one above and the other below the injection microdroplet on the same working dish by using PBS containing 1% FBS. After these microdroplets were spread over with mineral oil, the working dish was placed on the micromaniulator plate.

The cutting pipette installed on the micromanipulator was substituted with an injection pipette. The enucleated oocytes were washed three times with TCM199 washing medium and transferred into the injection microdroplet. The donor

cells were drawn up into the injection pipette and transferred into the injection microdroplet. Figure 4 shows the process of transferring a somatic cell into an enucleated oocyte. As shown in Figure 4, the enucleated oocyte was placed with its slit oriented toward the direction of 1 o'clock, fixed by using the holding pipette, and injected with the donor cell through the slit by employing the injection pipette and hydraulic pressure to give a reconstructed embryo. The embryo was washed three times with and incubated in TCM199 washing medium.

Example 3: Electrofusion and activation

The reconstructed embryos were subjected to electrofusion employing an Electrocell Manipulator(ECM 2001, BTX, USA), followed by activation. 15µl of mannitol solution containing 0.28M mannitol, 0.5mM HEPES(pH 7.2), 0.1mM $MgSO_4$ and 0.05% BSA was added to TCM199 culture medium containing the reconstructed embryos by employing a mouth pipette for washing. After 1 minute's incubation in the said medium, the embryos were incubated for 1 minute in mannitol solution supplemented with TCM199 washing solution, and finally transferred into mannitol solution by employing the mouth pipette for washing. The chamber(3.2mm chamber No. 453) of the Electrocell Manipulator was filled with mannitol solution supplemented with TCM199 washing medium, and then the embryos were placed in the chamber with their donor cell part facing the cathode. After the embryos were electrofused by applying DC pulse of 0.75 to 2.00kV/cm twice with one second's interval for 15µs each time, they were transferred into and washed three times with TCM199 washing medium by way of mannitol solution.

To activate the electrofused embryos, they were incubated in the dark for 4 minutes in ionomycin(Sigma Chemical Co., USA) solution which was TCM199 washing medium containing 5µM ionomycin and 1% BSA. The ionomycin stock solution was prepared by dissolving 1mg of ionomycin in

1.34 ml of DMSO. The activated embryos were incubated for 5 minutes in a 35mm dish containing TCM199 washing medium supplemented with 10% FBS to remove ionomycin from the embryos.

Example 4: Postactivation and in vitro culture of electrofused embryos

The activated embryos were postactivated for 4hrs in 25µl of cycloheximide(Sigma Chemical Co., USA) solution prepared by adding cycloheximide stock solution(10mg/ml in ethanol) to an in vitro culture medium, mTALP in a final concentration of 10 µg/ml. Then, the embryos were screened, and the selected embryos were incubated for 7 days under an environment of 39℃, 5% CO_2. During the culture, development of the embryos was monitored as time passed(see: Table 8).

Table 8: Development of embryos derived from human skin cells following inter-species nuclear transplantation

No. of elctrofused oocytes	Electrofusion rate(%)	Division rate (%)	No.(%) of 2-cell embryos	No.(%) of 8-cell embryos	No.(%) of morulae/ blastocysts
203	46.8	51.6	51.6	34.7	5.3

As shown in Table 8, it was clearly demonstrated that the inter-species nuclear transplantation technique makes possible the development of human cloned embryos to the morula/blastocyst stage, which eventually facilitates production of human embryonic stem cells from the developed morulae/blastocysts.

Based on the method described above, the inventors produced an embryo, SNU6(human somatic cell line), by using human skin cells as nuclear donors. The embryo was deposited with an international depositary authority,

KCTC(Korean Collection for Type Cultures; KRIBB #52, Oun-dong, Yusong-ku, Taejon, 305-333, Republic of Korea) on June 19, 2000 under an accession number of KCTC 0805BP.

Example 5: Freeze and thaw of embryos and transplantation

The embryos were frozen for long-term storage. First, a freezing medium(see: Tables 9 and 10) was distributed into 35mm dishes, and a freezer was turned on to be maintained at -5℃. The embryos selected for freezing were washed with PBS containing 10% FBS, and incubated in the freezing medium for 20 minutes. Then, the embryos were drawn up into a 0.25ml French straw to let the straw have the freezing medium containing the embryos in the middle and two layers of air at both ends. After the straw was heat-sealed by using a heated forcep, it was placed into the freezer, held at -5℃ for 5 minutes, and seeded with a forcep prechilled by liquid N_2. After seeding, the straw was cooled down at a rate of -0.3℃/min to -30℃, held for 10 minutes when the temperature reached -30℃. Finally, the embryos were stored in a liquid N_2 tank.

Table 9: Freezing PBS

Ingredient	Concentration
PBS(1×)	Gibco 14190-144
Na-pyruvate	0.033mM
Glucose	0.15mM
$CaCl_2 \cdot 2H_2O$	0.171mM
Penicillin-streptomycin	1% (penicillin 10000U/ml, streptomycin 10mg/ml)
$MgCl_2 \cdot 6H_2O$	0.049mM

Table 10: Freezing medium

Ingredient	Concentration
Freezing PBS(Table 9)	2.25ml(45%)
Fetal bovine serum(FBS)	2.25ml(45%)
Glycerol	0.5ml(10%)

To thaw the frozen embryos, a thawing medium containing PBS supplemented with 20% FBS was prepared in 35mm dishes, and added with glycerol to give thawing media each having 0%, 3% and 6% glycerol(see: Tables 9 and 11). Then, the frozen straw was taken out from the liquid N_2, held in the air for 5 seconds, and thawed in a container(>20cm in diameter) containing warm water(30℃). After thawing, the straw was cut on the air layers at both ends, and the medium containing the embryos was collected. The embryos were examined under the microscope. To remove the freezing medium from the embryos, they were consecutively incubated in the thawing media containing 6% glycerol, 3% glycerol and 0% glycerol, each for 5 minutes.

Table 11: Thawing media

Ingredient	6% Glycerol PBS	3% Glycerol PBS	0% Glycerol PBS
PBS	(Table 9)	(Table 9)	(Table 9)
BSA	0.5%	0.5%	0.5%
Glycerol	6%	3%	0%
Sucrose	0.3M	0.3M	0.3M

As clearly illustrated and explained above, the present invention provides a method for producing human cloned embryos by inter-species nuclear transplantation technique involving transfer of human somatic cell-derived nuclei into oocytes obtained from cow, and human cloned embryos produced by the said method. In accordance with the method of the invention, production of human cloned embryos can be employed to obtain human embryonic stem cells which may be widely applied for various purposes such as disease cure and other applications in pharmaceutics and medical science.

Various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing descriptions. Such modifications are also intended to fall within the scope of

the appended claims.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in description
On pages 9 , lines 1-5 .

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on additional sheet ☐

Name of depositary institution

Korean Collection for Type Cultures(KCTC)

Address of depositary institution (including postal code and country)

Korean Collection for Type Cultures(KCTC)
KRIBB #52, Oun-dong, Yusong-ku
Taejon, 305-333, Republic of Korea

Date of deposit	Accession Number
June 19, 2000	KCTC 0805BP

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information continues on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g.. "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

WHAT IS CLAIMED IS:

1. A method for producing human cloned embryos which comprises the steps of:

(i) preparing donor somatic cell lines collected from human;

(ii) maturing oocytes collected from ovary of cow in vitro;

(iii) removing cumulus cells surrounding the oocytes, cutting a portion of zona pellucida of the matured oocytes to make a slit, and squeezing out a portion of cytoplasm including the first polar body through the slit to give enucleated recipient oocytes;

(iv) transferring a nucleus to the recipient oocyte by injection of the donor cells to the enucleated recipient oocytes, followed by the subsequent electrofusion and activation of the electrofused cells to give embryos;

(v) postactivating and culturing the embryos in vitro.

2. The method for producing human cloned embryos of claim 1, wherein the somatic cell lines prepared in Step(i) include human skin cells or fibroblasts collected from umbilical cord of newborns.

3. The method for producing human cloned embryos of claim 1, wherein the somatic cell lines are stored by subculture, serum starvation culture or freezing.

4. The method for producing human cloned embryos of claim 1, wherein the cumulus cells surrounding the oocytes in Step (iii) are physically removed with a denuding pipette after treatment of hyaluronidase.

5. The method for producing human cloned embryos of claim 1, wherein the enucleation of oocytes in Step(iii) is carried out by making a slit on the oocyte by cutting it with micromanipulator; placing the oocyte with its slit

oriented vertically and holding a lower part of the oocyte with a holding pipette to prevent the cell from moving; squeezing the upper part of the oocyte with a cutting pipette to let 10 to 15% of cytoplasm containing the first polar body out of the oocyte through the slit.

6. The method for producing human cloned embryos of claim 1, wherein the nuclear transfer in Step(iv) is carried out by injecting a donor cell into a recipient enucleated oocyte through the slit made on zona pellucida of the oocyte.

7. The method for producing human cloned embryos of claim 1, wherein the electrofusion in Step(iv) is carried out by applying DC pulse of 0.75 to 2.00kV/cm twice with one second's interval for 15μs each time.

8. The method for producing human cloned embryos of claim 1, wherein the activation in Step(iv) occurs in a simultaneous manner with electrofusion provided that the electrofusion is performed in a medium containing Ca^{2+}.

9. The method for producing human cloned embryos of claim 1, wherein the activation in Step(iv) is performed in ionomycin solution in the dark provided that the electrofusion is carried out in a Ca^{2+}-free medium.

10. The method for producing human cloned embryos of claim 1, wherein the postactivation in Step(v) is carried out by culturing embryos in cycloheximide solution or DMAP(4-dimethylaminopurine) solution.

11. The method for producing human cloned embryos of claim 1, wherein in vitro culture in Step(v) is carried out by culturing the postactivated embryos in mTALP, mSOF or mCR2aa medium.

12. The method for producing human cloned embryos of claim 1, further comprising a step of storing embryos cultured in vitro in Step(v) for later use after freezing the embryos in a freezing medium containing penicillin-streptomycin, $CaCl_2$, glucose, $MgCl_2$, Na-pyruvate and phosphate buffered saline.

13. An embryo, SNU6(human somatic cell line, KCTC 0805BP), which is produced by method of claim 1 employing human skin cells and oocytes of Korean cow as nucleus donors and recipient oocytes, respectively.

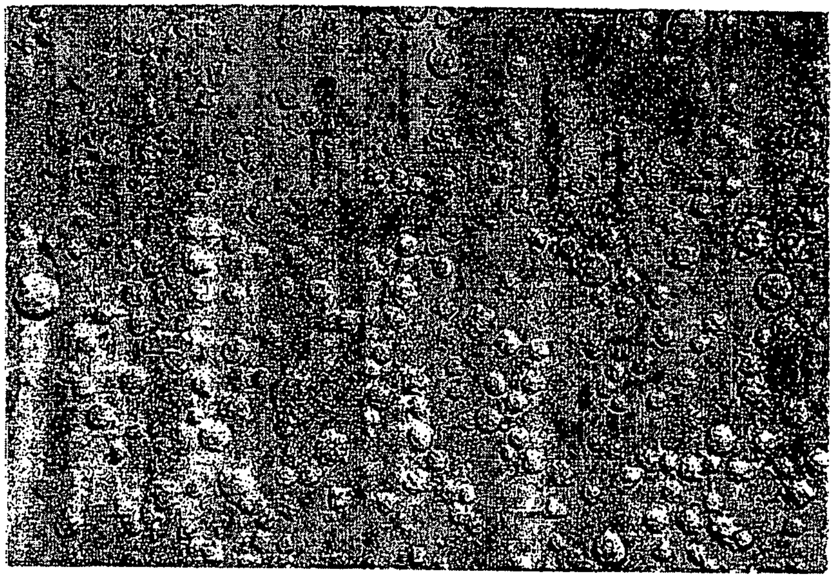

Fig. 1

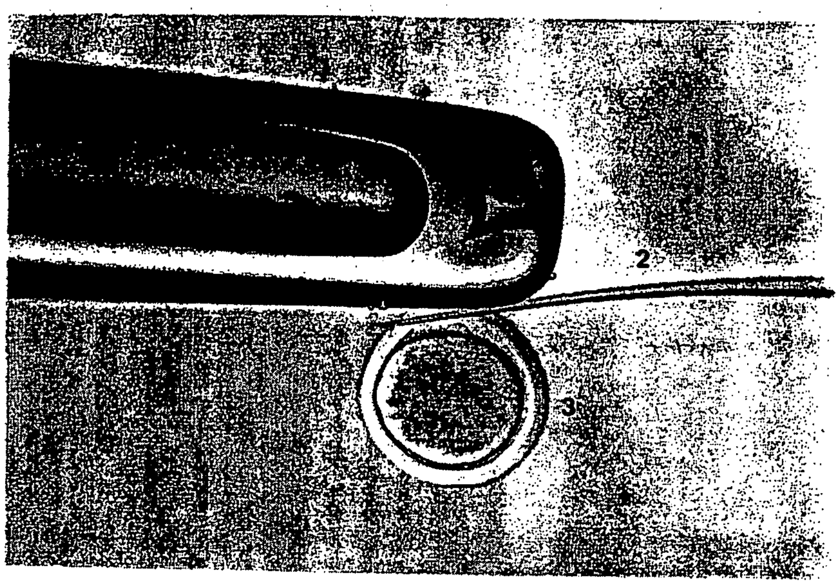

Fig. 2

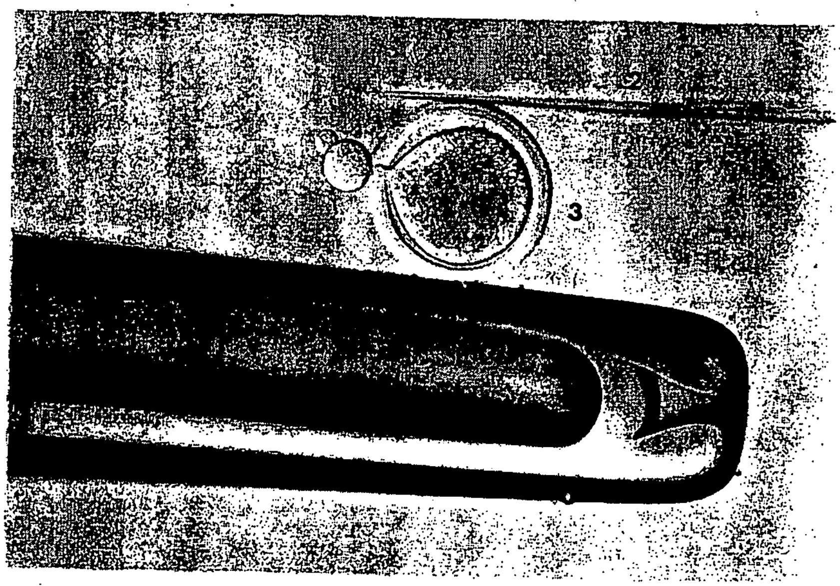

Fig. 3

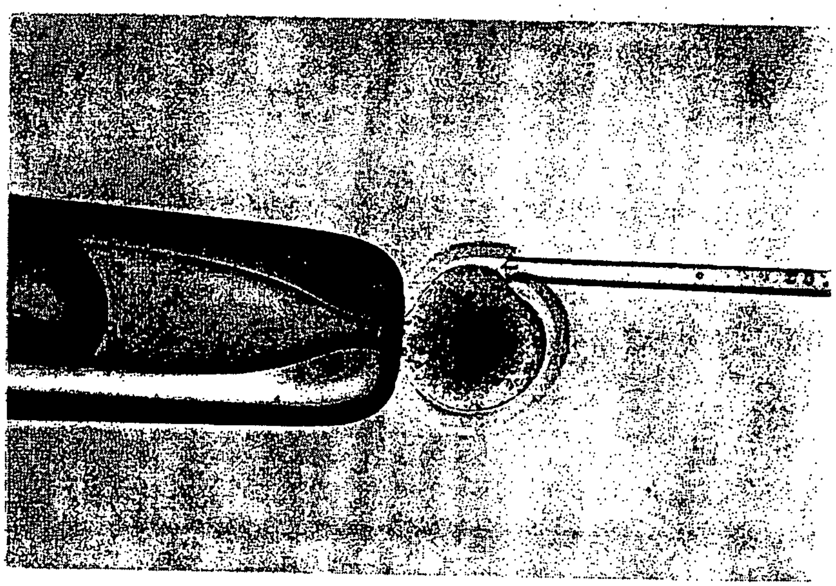

Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00705

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)
IPC 7 C12N 5/00

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)
NCBI pubmed, IBM patent database, USPTO patent database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9707668 A (Roslin Institute) 6 Mar 1997 (06. 03. 97) column 21-24	1-13
A	WO 9707669 A (Roslin Institute) 6 Mar 1997 (06. 03. 97) column 21-34	1-13
P	US 6011197 A (Infigen Inc.) 4 Jan 2000 (04. 01. 00)	1-13
A	US 5945577 A (University of Massachusetts) 31 Aug 1999 (31. 08. 99)	1-13
A	Nature Mar 1996, vol 380, pages 64-66	1-13
A	Nature Feb 1997, vol 385, pages 810-813	1-13
A	Nature 1998, vol 394, pages 369-374	1-13
A	J. Reprod. Fert. 1992, vol 96, pages 725-734	1-13

☐ Further documents are listed in the continuation of Box C.   ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 SEPTEMBER 2000 (28.09.2000)	29 SEPTEMBER 2000 (29.09.2000)
Name and mailing address of the ISA/KR Korean Industrial Property Office Government Complex-Taejon, Dunsan-dong, So-ku, Taejon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer LIM, Hea Joon Telephone No. 82-42-481-5590

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR00/00705

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9707668	06-03-97	EP-A- 0847237	17-06-98